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I, LEANNE MYNOTT, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 8658 for a patent by THE UNIVERSITY OF MELBOURNE filed on 12 February 1999.

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L. Mynott

LEANNE MYNOTT
TEAM LEADER EXAMINATION
SUPPORT AND SALES

Regulation 3.2

The University of Melbourne

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A method of treatment and agents useful for same"

The invention is described in the following statement:

A METHOD OF TREATMENT AND AGENTS USEFUL FOR SAME

FIELD OF THE INVENTION

The present invention relates generally to a method of inducing, stimulating or otherwise facilitating bronchoprotection in humans and animals by modulating bronchial constriction and/or inflammation. The present invention is predicated in part on the identification of receptors in airway epithelium which mediate inhibition of bronchoconstriction and/or inflammation following their activation. More particularly, the present invention identifies that activation of protease activated receptors (PARs) results in relaxation of airway epithelium. Activation of airway epithelium PARs inhibits bronchoconstriction and/or inflammation and thereby mediates bronchoprotection of the airways. The present invention further provides a method for the prophylaxis and treatment of disease conditions in airways such as asthma and bronchitis and further provides methods for the diagnosis and screening of agents useful in the prophylaxis and treatment of airway disease conditions.

BACKGROUND OF THE INVENTION

The subject specification contains amino acid sequence information prepared using the programme PatentIn Version 2.0, presented herein after the bibliography. Each amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (e.g. protein (PRT), etc) and source organism for each amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (eg. <400>1, <400>2, etc).

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Many receptors for biologically-active effector molecules are large proteins embedded in biological membranes. They serve as transducers of information mediated by effectors such as hormones and cytokines, and are also important in the mechanism of action of pharmaceutical agents. For example, receptors located within the outer regions of the cellular membrane act to transduce such information into the cell, which may then respond in a number of different ways *via* specific secondary messenger systems. Therefore, these types of receptors have specific extracellular and intracellular domains which allow information, such as hormonal signals, to be appropriately detected and processed by cells.

Protease-activated receptors (PARs) are a relatively new subtype of a superfamily of membrane receptors which have seven membrane-spanning regions and are coupled to intracellular second messenger mechanisms *via* G proteins. The three known members, respectively designated PAR-1, PAR-2 and PAR-3, have been cloned, and shown to be expressed in vascularised tissues comprising endothelial and smooth muscle cells (PAR-1 and PAR-2) and platelets (PAR-1 and PAR-3). A fourth receptor, designated PAR-4, has also recently been demonstrated on platelets of PAR-3 deficient mice and has been cloned (Kahn *et al,* 1998); the human homologue has also been cloned (Xu *et al,* 1998).

PARs are activated in a unique manner, which is illustrated in Figure 1. As the name indicates, limited proteolysis by specific proteases (proteinases) removes part of the extracellular N-terminal region of the receptor, so that the newly-shortened N-terminal acts as a ligand for an as yet undefined binding region on the remainder of the receptor in order to signal the cell to respond. Thus, PARs have their own inbuilt or "tethered" ligands, and the specific protease activity reveals that these latent, intrinsic ligands act as ligands in their own right rather than as exogenous effectors.

PAR-1 (Vu *et al,* 1991; Coughlin *et al,* 1992) and PAR-3 (Ishihara *et al,* 1996) are activated primarily by the blood-borne protease, thrombin, which is believed to be involved in thrombosis, inflammation and mitogenic growth (De Caterina & Sicari, 1993; Dennington & Berndt, 1994; Fager, 1995). For example, thrombin causes smooth muscle in the airways to proliferate, which may cause the airway to thicken and become obstructed. PAR-1 is also

located on vascular endothelial cells, where, like many other receptor types, stimulation leads to release of nitric oxide (NO) and other factors which then cause the muscle in the wall of the vessels to relax (Muramatsu *et al,* 1991; Tesfamariam *et al,* 1993; Tesfamariam, 1994; Hwa *et al,* 1996; Saifeddine *et al,* 1996). Under normal circumstances, the enzymatic activity of thrombin is strongly suppressed by a number of endogenous inhibitors.

PAR-2 differs from both PAR-1 and PAR-3 receptors in that it is activated not by thrombin, but by trypsin and trypsin-like enzymes, such as mast cell-derived tryptase (Molino *et al,* 1997). Trypsin is usually confined to the upper gastrointestinal tract after its generation by activation of its pancreatic precursor, trypsinogen. Trypsinogen is induced in vascular endothelial cells by tissue plasminogen activator [TPA] (Koshikawa *et al,* 1997). Tryptase is released in large concentrations from mast cells (Caughey, 1994). Mast cells are believed to have a central role in the pathogenic manifestations of asthma. Tryptase stimulates mucus release and can inactivate some peptides such as vasoactive intestinal peptide (VIP) that relax airway smooth muscle in experimental animals. This suggests that the PARs play a role in the aetiology of airway disease by inducing contraction of smooth muscle cells.

In addition to tryptase, tryptase-like enzymes are released by clara cells (Yasuoka *et al,* 1997), which are common in the epithelium lining the small bronchi of most mammals, including humans, the trachea of the mouse, and by lymphocytes which enter the inflamed airway in large numbers. Trypsin has been localised to normal airway epithelium (Koshikawa *et al,* 1997). In addition, tryptase-like enzymes are thought to be involved in a number of inflammatory responses and diseases, such as atherosclerosis (Atkinson *et al,* 1994; Kovanen *et al,* 1995) and varicosis (Yamada *et al,* 1996). Furthermore and importantly, as well as directly activating mast cell degranulation *via* IgE-antigen recognition, the antigens of some dust mites and pollens are proteases with trypsin-like activity (Caughey, 1997). Therefore, allergens which are central to, and the causal agents of, many airway diseases have the potential to directly and indirectly activate PAR-2.

PAR-1 and PAR-2, but not PAR-3 (Isihara *et al,* 1997) can also be activated by short synthetic peptide sequences corresponding to those of the tethered ligands. For PAR-1, this

tethered ligand is SFLLRN-NH_2 (single amino acid code [<400>1], which is also known as TRAP (thrombin receptor-activating peptide)). The tethered ligand sequence for mouse PAR-2 is SLIGRL-NH_2 [<400>2], and is referred to herein as PAR-2 activating peptide (PAR-2-AP). Therefore, these peptides can be used to mimic enzyme mediated PAR activation and to study the effects of PAR activation.

The genes for PAR-1, PAR-2 and PAR-3 have been cloned (Vu *et al,* 1991; Nystedt *et al,* 1994; Bohm *et al,* 1996a; Saifeddine *et al,* 1996 and Ishihara *et al,* 1997). PAR-2 mRNA has been shown to be highly expressed in vascularised or endothelialised tissues such as the stomach, intestine, pancreas, kidney and liver. In the gut, PAR-2 mRNA is located mainly in epithelial cells (Bohm *et al,* 1996b). In blood vessels, functional PAR-2 has been localised nearly exclusively to endothelial cells, where, like PAR-1, it mediates endothelium-dependent vasodilation (Hwa *et al,* 1996; Saifeddine *et al,* 1996). It has been proposed that PAR-2 acts as a trypsin sensor in the pancreas (Bohm *et al,* 1996a) and is involved in a possible cytoprotective mechanism for gut epithelia exposed to trypsin (Bohm *et al,* 1996b). Apart from these proposed activities, little is known of other physiological roles for these receptors.

Following activation, PARs are inactivated by rapid internalisation, which also provides the signals for rapid generation of new receptors from intracellular pools and *de novo* protein synthesis (Hoxie *et al,* 1993; Bohm *et al,* 1996b). This provides a powerful self-replenishing system to maintain adequate tissue levels of the receptors.

~~Like PAR-1, PAR-2 mediates relaxation of arteries *via* the release of nitric oxide (NO;~~ Moncada *et al,* 1991) and of endothelium-derived hyperpolarising factor (EDHF: Garland *et al,* 1995), although the EDHF-dependent mechanism for PAR-1 is different from that for PAR-2. The mechanisms of receptor recycling also regulate the way in which endothelial cells recover their ability to respond to further protease challenge, at least within two to three hours after the first challenge. For PAR-1, this recovery process involves rapid recycling of receptors (30 min- 150 min) without the tethered ligand sequence, but no new N-terminal receptors are produced. For PAR-2, however, fully intact new receptors are rapidly synthesised from stable mRNA, and are inserted into the plasma membrane (Bohm *et al,*

1996a).

Only PAR-1 has been identified in the human vasculature (Nelken, 1992), where expression was reported to be isolated to endothelial cells in atheroma-free arteries. In vessels affected by atherosclerosis, PAR-1 mRNA was found in endothelial, smooth muscle and mesenchymal-appearing cells. Studies on human endothelial cell PAR function have been limited to the measurement of calcium fluxes in transfected cell lines (Mari, 1996) and umbilical vein endothelial cells (Ngaiza, 1991; Kruse, 1995). An atypical PAR has also been identified in human coronary arteries (Hamilton *et al,* 1998).

The incidence and prevalence of airway diseases such as asthma and bronchitis, which are characterised by airflow obstruction, inflammation and pathological changes in airway tissue are increasing globally (Barnes *et al,* 1996a). However, it is unknown why some people develop these types of airway diseases, while other people exposed to the same environmental factors do not. One possibility is that the airway defences of patients who develop the disease are less efficient than those of non-afflicted subjects.

Asthmatic patients suffer from episodic airflow limitation caused by bronchospasm, oedema and thickening of the airway walls. In addition, one of the hallmarks of asthma is that the bronchi are hypersensitive to specific and non-specific stimuli, causing them to contract too much and too sensitively, thereby narrowing the airways and making breathing difficult (Barnes, 1996; Barnes *et al,* 1996b). The most widely-used treatment for asthma is administration of drugs that cause the bronchial muscles to relax and the airways to dilate, thus restoring the ability to breath. The most commonly used drugs for this purpose are the so-called beta-2 agonists. These drugs stimulate another subtype of the seven transmembrane, G protein-coupled receptor superfamily, the beta-2 adrenoceptors, which are located on the muscle and mediate relaxation *via* well-defined biochemical mechanisms. While beta-2 agonists are effective in most patients, it has recently been discovered that some asthmatics respond poorly to beta-2 agonists, and the agonists may mediate down-regulation of patient responses during chronic treatment due to genetic mutations in the beta-2 adrenoceptor sequence. Additionally, concerns have been raised about the possibility that

regular use of beta 2-adrenoceptor agonists may increase the risk of death from asthma.

Airway disease like asthma and bronchitis are predicted to continue their dramatic rate of increase in developed societies, and therefore, new therapies, procedures and methods of diagnosis, and methods of screening for prophylactic or therapeutic agents are urgently needed.

In work leading up to the present invention, the inventors identified that activation of PARs located immunohistochemically on airway epithelium, caused dilation of bronchi and bronchioles. This physiologically relevant protective response in airways was mediated mainly by a cyclooxygenase product (eg. PGE_2) released from the epithelium. In addition, after receptor desensitisation due to internalisation and degradation, functional PARs are rapidly replenished to the cell surface by protein trafficking and *de novo* synthesis. In accordance with the present invention, epithelial PARs, and in particular PAR-2 are regarded as potential targets for the development of new therapies for inflammatory diseases like asthma and bronchitis.

The inventors have now surprisingly found that PAR-2 in the epithelial layer has an anti-inflammatory role in the airways, and that PAR-2 epithelial and smooth muscle cells are differentially regulated.

The inventors have shown that epithelial PARs and in particular PAR-2, initiate important autocrine and paracrine protective tissue responses in the airways which include regulation of smooth muscle contractility, inflammatory cell migration and function, neural activity and tissue remodelling, and therefore enable new therapies for airway inflammatory diseases like asthma and bronchitis.

Aspects of work leading up to the present invention have been disclosed in Australian Patent Application No. PP5922 filed 15 September, 1998 which is incorporated herein by reference.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

One aspect of the present invention provides an isolated molecule comprising protease activated receptor (PAR) activity wherein said molecule is isolatable from airway epithelium and upon activation, stimulates, induces or otherwise facilitates inhibition of bronchoconstriction and/or inflammation in humans and animals.

Another aspect of the present invention is directed to an isolated molecule comprising protease activated receptor-2 (PAR-2) activity wherein said molecule is isolatable from airway epithelium and upon activation, stimulates, induces or otherwise facilitates inhibition of bronchoconstriction and/or inflammation in humans and animals.

Yet another aspect of the present invention provides a recombinant, synthetic or purified, naturally occurring molecule comprising protease activated receptor -2 (PAR-2) activity wherein said molecule in its naturally occurring form is isolatable from airway epithelium and upon activation by a PAR-2 activating peptide, stimulates, induces or otherwise facilitates inhibition of bronchoconstriction and/or inflammation in humans and animals.

Still yet another aspect of the present invention contemplates a method for the prophylaxis or treatment of an airway disease condition in a human or animal said method comprising administering to said human or animal an effective amount of an agent capable of activating an airway epithelium protease activated receptor (PAR) for a time and under conditions sufficient for activation of said PAR which then stimulates, induces or otherwise facilitates inhibition of bronchoconstriction and/or inflammation.

Even yet another aspect of the present invention contemplates a method for the prophylaxis

or treatment of an airway disease condition in a human or animal said method comprising administering to said human or animal an effective amount of an agent capable of activating an airway epithelium protease activated receptor-2 (PAR-2) for a time and under conditions sufficient for activation of said PAR which then stimulates, induces or otherwise facilitates inhibition of bronchoconstriction and/or inflammation.

Another aspect of the present invention is directed to a composition useful for facilitating bronchoprotection said composition comprising an activator of a protease activated receptor (PAR) in airway epithelium and one or more pharmaceutically acceptable carriers and/or diluents.

Although the present invention is particularly directed to inhibition of bronchoconstriction and/or inflammation, the subject invention extends to PARs such as PAR-2 orchestrating a wide range of bronchoprotective responses. In particular, the present invention extends to the development of technology stemming from the recognition that endogenous activators (e.g. trypsin) and PARs (e.g. PAR-2) are co-localised in the epithelium and act as an amplifier of a PAR (e.g. PAR-2) protective mechanism.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation of PAR-2. The black loops depict the membrane-spanning regions in a theoretical cell. The receptor is activated by trypsin (or by other ~~trypsin-like proteases, eg. tryptase) by cleavage of the arginine^{34}-serine^{35} peptide bond amino~~ terminally to the arginine^{34} in the extracellular N-terminal domain. The next approximately six amino acids of the new N-terminal (called the tethered ligand sequence, solid box) now "flip" on to another, undefined region of the remaining receptor to initiate intrallecular G-protein (G) coupling and signalling, shown here as "responses". The putative tethered ligand binding region ("R") of the receptor can also be directly activated by exogenous addition of a synthetic peptide identical to the tethered ligand sequence SLIGRL-NH_2 (single letter amino acid code [<400>2] designating the mouse PAR-2 activating sequence. The similar but genetically distinct PAR-1, or thrombin receptor, is enzymically activated by thrombin by

cleaving a arginine[41]-serine[42]-bond and the synthetic tethered ligand sequence, SFLLRN-NH [<400>1] designating the human PAR-2 activating sequence.

Figure 2 are photographic and diagrammatic representations showing immunohistochemical localisation of PAR-2 in mouse bronchi and demonstration that PAR-2 and PAR-1 mediate epithelium-dependent relaxation in isolated rings of this tissue. (a) Confocal photomicrograph showing PAR-2 immunofluorescence in discrete epithelial cells (arrow) as well as smooth muscle cells (m) and fibroblasts (arrow head). In some epithelial cells, the fluorescence appeared concentrated within areas of the cytoplasm. Pre-absorption with the peptide sequence used to raise the mouse PAR-2 antibody quenched the epithelial, smooth muscle and fibroblast fluorescence. The scale bar represents 10 μm. (b) An original, digitised chart recording of changes in isometric force in a single ring of mouse left bronchus with intect epithelium. The tissue was contracted to approximately 40% F_{max} to acetylcholine (ACh; 30μM - see Examples) with cumulative, titrated concentrations of carbachol (note the change of gain and that force recovered spontaneously over the 15 min break in the trace of maximum relaxation to SLIGRL-NH_2 [<400>2]. (c) Removal of the epithelium with 0.1% v/v Triton X-100 (see Examples) abolished relaxations to SLIGRL-NH_2 [<400>2] and SFLLRN-NH_2 [<400>1] whereas the tissue could still sensitively relax to PGE_2. (d) Light photomicrographs of cross sections of mouse bronchi showing that the 0.1% v/v Triton X-100 perfusion technique removed the vast majority of columnar epithelial cells (arrows) with no microscopic evidence of damage to the underlying smooth muscle (m). Scale bar represents 30 μm.

Figure 3 are graphical representations showing mechanisms of PAR-mediated bronchial relaxation. (a) Epithelium- and (b) cyclooxygenase-dependent relaxations of mouse bronchi to the PAR-2 and PAR-1 synthetic peptide ligands, SLIGRL-NH_2 [<400>2] and SFLLRN-NH_2 [<400>1], respectively. (c) Relaxations to trypsin and thrombin in epithelium-intact preparations were similarly abolished by cyclooxygenase inhibition. Group data from similar experiments as that described in Figure 2 except tissues were either treated or not treated with indomethacin (3 μM) and aspirin (100 μM) to block cyclooxygenase activity or a combination of the NO inhibitors L-NOARG (100 μM) and oxyhaemoglobin (20 μM). All

relaxations and contractions are expressed as percentages of the initial force to carbachol (40% F_{max}) regardless of treatment. Values on the graphs are mean ± s.e. mean from 5-9 experiments, except aspirin (n=3). * P<0.01. Note that the NO inhibitors had no effect on the relaxations to PAR-1 and PAR-2-activating peptides. Note: (O) represents control response in all cases. In (a) (▲) represents removal of the epithelium and in (b) inhibition of NO. (▼) and (■) represent treatment with aspirin and indomethacin respectively.

Figure 4 are graphical representations showing recovery of responsiveness to PAR-2 activation after trypsin desensitisation in the mouse bronchus. (a) Responsiveness to trypsin (0.3 U/ml) recovered to approximately 70% of control levels 45 min after the desensitising concentration of trypsin (0.3 U/ml) was washed from the bath (see Methods). Time control responses to trypsin at 15, 45, 80 and 120 min recovery were not significantly different from the initial control. (b) The recovery of trypsin sensitivity at 45 min was abolished by the protein trafficking inhibitor, brefeldin A (10 μM) or the translation inhibitor cyclohexmide (70 μM). Neither compound had any effect on time control responses to trypsin. Values are mean ± s.e. means from 3-12 experiments.

Figure 5 are graphical representations demonstrating that the PAR-2 activating peptide SLIGRL-NH_2 [<400>2] causes inhibition of bronchoconstriction *in vivo*. Original chart recordings (a, b) and grouped data (c, d) showing the effect of a 30 sec exposure to an aerosol of a 0.1 mg/ml solution of SLIGRL-NH_2 [<400>2] on 5-HT (3nmol/kg i.v)-induced changes in airway resistance (R_L; a, c) and dynamic compliance (C_{dyn}; b, d) in the anaesthetised rat. Not shown is the complete inhibition of bronchoconstriction to 5-HT lasting at least 45 min occurred when SLIGRL-NH_2 [<400>2] was used at 1 mg/ml. Values are mean ± s.e. from n=3 experiments.

Figure 6 is photographic representation showing dual immunohistochemical localisation of PAR-2 (green) and trypsin(ogen) (red) within the epithelium of a 3 μm thick cross section of humanintrapulmonary bronchiole. Note that in some cells PAR-2 and trypsin (ogen) are co-expressed. Scale bar represents 20 μm.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on the identification of airway epithelial PARs which modulate bronchodilation and inflammation. More particularly, the inventors have identified PARs in airway epithelium which, upon activation, simulate, induce or otherwise facilitate inhibition of bronchoconstriction and/or inflammation in humans an animals.

Accordingly, one aspect of the present invention provides an isolated molecule comprising PAR activity wherein said molecule is isolatable from airway epithelium and upon activation, stimulates, induces or otherwise facilitates inhibition of bronchoconstriction and/or inflammation in humans and animals.

Reference herein to animals includes apart from humans, primates, livestock animals (e.g. sheep, cows, horses, pigs, goats), laboratory test animals (e.g. mice, rats, rabbits, guinea pigs), companion animals (eg. cats, dogs) and captive wild animals (e.g. foxes, deer, kangaroos).

Although the present invention extends to any PAR expressed in airway epithelium, it is particularly directed to PAR-1 and PAR-2 and is most particularly directed to PAR-2. Accordingly, reference hereinafter to "PAR-2" includes other PARs which behave in a functionally similar manner.

Another aspect of the present invention is directed to an isolated molecule comprising PAR-2 activity wherein said molecule is isolatable from airway epithelium and upon activation, stimulates, induces or otherwise facilitates inhibition of bronchoconstriction and/or inflammation in humans and animals.

Preferably, the PAR-2 is in isolated form meaning that it is has undergone at least one purification step away from contaminating material. However, PAR-2 may also be part of a membrane formulation or preparation. PAR-2 may also be prepared in recombinant form or be chemically synthesized.

The recombinant form of PAR-2 may be as a single polypeptide or a modular molecule comprising various parts of PAR-2 or its homologues.

Accordingly to this aspect of the present invention there is provided a polypeptide in recombinant form which is homologous to a PAR-2 in airway epithelium, said polypeptide comprising an N-terminal portion, transmembrane portion, an intracellular portion and a ligand binding portion wherein upon proteolytic cleave of the N-terminal portion, the remaining extracellular portion folds onto or otherwise interacts with the ligand binding portion to activate the recombinant polypeptide.

Each portion of the above polypeptide may be derived from airway epithelium PAR-2 or it may be in modular form meaning that the portions are derived from different molecules. For example, the extracellular portion may be from PAR-2, the transmembrane portion may be from another receptor and the intracellular portion may be any G-protein interacting region.

Yet another aspect of the present invention provides a recombinant, synthetic or purified, naturally occurring molecule comprising PAR-2 activity wherein said molecule is isolatable from airway epithelium and upon activating by a PAR-2 actuating peptide stimulates, induces or otherwise facilitates inhibition of bronchoconstriction and/or inflammation in humans and animals.

The identification of PAR-2 airway epithelium provides a mechanism for treating airway ~~disease conditions which result in bronchoconstriction and/or inflammation of airway tissue.~~

In a preferred embodiment, the condition to be treated is a broncho-constrictive disease such as but not limited to asthma, bronchitis including brochiolitis obliterans, rhinitis, hayfever, alreolitis of diverse aetiologies, ciliary dyskinesin sarcoidosis and pulmonary inflammatory diseases.

According to this aspect of the present invention, there is contemplated a method for the prophylaxis or treatment of an airway disease condition in a human or animal said method

comprising administering to said human or animal an effective amount of an agent capable of activating an airway epithelium PAR for a time and under conditions sufficient for activation of said PAR which then stimulates, induces or otherwise facilitates inhibition of bronchoconstriction and/or inflammation.

Preferably, the PAR is PAR-2.

According to this preferred embodiment, there is provided a method for the prophylaxis or treatment of an airway disease condition in a human or animal said method comprising administering to said human or animal an effective amount of an agent capable of activating an airway epithelium PAR-2 for a time and under conditions sufficient for activation of said PAR-2 which then stimulates, induces or otherwise facilitates inhibition of bronchoconstriction and/or inflammation.

The agent may be a nucleotide sequence, low molecular weight compound, or a derivative, part, fragment, analogue, mimetic, mimotope or chemical equivalent of all or a portion of PAR-2. In particular, the agent may be a peptide having similar biological activity to SFLLRN-NH_2 [<400>1], SLIGRL-NH_2 [<400>2] and/or SLIGKV-NH_2 [<400>3] (see Blackhart *et al,* 1996). Gene therapy may also be employed such as using cationic liposomes for gene vector transfer.

The compositions may be administered orally, intranasally, *via* aerosol, *via* inhalation, parenterally, intreperitoneally, intravenously, rectally or subcutaneously amongst other routes. Gene transfer vectors may also be employed.

Accordingly, another aspect of the present invention is directed to a composition useful for facilitating bronchoprotection said composition comprising an activator of PARs in airway epithelium and one or more pharmaceutically acceptable carriers and/or diluents.

The activator of this aspect of the present invention may be referred to as an "active ingredient" or "agent".

Compositions suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. They are generally stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyoil (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by, for example, filter sterilization or sterilization by other appropriate means. In the case of sterile powders for the preparation of sterile injectable solutions, a preferred method of preparation includes vacuum drying and freeze-drying which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution.

When the active ingredient is suitably protected, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; and a lubricant such as magnesium stearate. Any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

Effective amounts of the subject agent will vary depending on the condition to be treated by may range from 0.001 ng/kg body weight to 100 mg/kg body weight. The agent may be administered every minute or hourly, daily, weekly or monthly. The agent may be used prophylactically or in the treatment of a disease condition.

Methods and pharmaceutical carriers for preparation of pharmaceutical compositions are well known in the art, as set out in textbooks such as Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

Another aspect of the present invention relates to a method of diagnosis of a condition mediated by bronchial contraction, comprising the step of activating a PAR as described above and measuring cellular response(s). The types of response(s) induced can be used as an indicator of pre-disposition to one or more of the conditions described above, thereby enabling diagnosis.

This method also enables the screening of putative therapeutic or prophylactic agents for one or more of these conditions. Accordingly, another aspect of the present invention provides a method of screening putative agents for the treatment or prophylaxis of a direct or indirect condition mediated by changes in smooth muscle cell contractility, comprising the step of exposing a PAR to the putative agent and measuring the ability of the agent to activate the PAR. Preferably, the PAR is PAR-1 or PAR-2 or a PAR-like receptor.

The present invention further extends to antibodies to PAR-2 and in particular to extracellular portions of PAR-2. Such antibodies may be monoclonal or polyclonal. The antibodies of

the present invention are particularly useful for as therapeutic (e.g. as antagonists) or as diagnostic agents.

PAR-2 of the present invention may be used, for example, as an antigen to screen for naturally occurring antibodies to PAR-2 in humans or animals. Alternatively, specific antibodies to PAR-2 may be used to screen for PAR-2 or an antigenic derivative or relative in a sample. This may provide an indication of whether PAR-2 is immunologically normal and, if not, this may indicate a propensity to develop airway disease. Techniques for such assays are well known in the art and include, for example, sandwich assays and ELISA.

Accordingly, the present invention provides a method for detecting the presence of a PAR-2 or an antigenic fragment thereof in a biological sample, said method comprising contacting said biological sample with an antibody to said PAR-2 for a time and under conditions sufficient for a complex to form between said PAR-2 and an antibody and then detecting said complex.

A biological sample according to this aspect is one which potentially contains PAR-2 containing cells such as flem, respiratory mucus or biopsy tissue. In this context, a biological sample includes tissue and tissue extract. The presence of PAR-2 in a biological sample may be determined using a wide range of immunoassay techniques such as those described in US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. This includes both single-site and two-site, or "sandwich", assays of the non-competitive types, as well as in the traditional competitive binding assays. Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay techniques exist, and all are intended to be encompassed by the present invention.

Reference herein to "PAR" and more particularly "PAR-2" includes all derivatives, mutants, parts, fragments, portions, homologues, mimetics, mimotopes, analogues or chemical equivalents of all or part of PAR-2.

Analogues and mimetics include molecules which contain non-naturally occurring amino

acids as well as molecules which do not contain amino acids but nevertheless behave functionally the same as PAR-2. Natural product screening is one useful strategy for identifying analogues and mimetics. Natural product screening involves screening environments such as bacteria, plants, animals, rainforests, riverbeds, seabeds, aquatic environments, coral and antarctic or arctic environments for naturally occurring molecules which mimic, agonise or antagonise the subject NP of the present invention. Analogues of the subject PAR-2 contemplated herein include modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide synthesis and the use of crosslinkers and other methods which impose conformational constraints on the peptide molecule or their analogues.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid contemplated herein is shown in Table 1.

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle

	D-tryptophan	Dtrp	L-norvaline	Nva
	D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
	D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
	D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
5	D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
	D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
	D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
	D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
10	D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
	D-α-methylleucine	Dmleu	α-napthylalanine	Anap
	D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
	D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
15	D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
20	D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
25	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
30	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis

D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe

N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_α-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

All these types of modifications may be important to stabilise PAR-2 or a PAR-2 modulating agent. This may be important if these molecules are used, for example, in the manufacture of a therapeutic or diagnostic composition.

The present invention further contemplates chemical equivalents of the subject polypeptides. ~~Chemical equivalents may not necessarily be derived from the subject PAR-2 itself but may~~ share certain conformational or functional similarities. Alternatively, chemical equivalents may be specifically designed to mimic certain physiochemical properties of the polypeptides. Chemical equivalents may be chemically synthesised or may be detected following, for example, natural product screening.

Reference herein to the PAR-2 of the present invention should be read as including reference to all forms of the PAR-2 including, by way of example, isoforms, monomeric, dimeric and multimeric forms and peptide fragments PAR-2 as well as other PARs.

EXAMPLE 1

In vitro studies

The right and left main bronchi and their first order branches of specific pathogen-free (SPF) Balb/c mice (15-20 g; either sex), Hartley tricolour guinea-pigs (300-400 g; male) and Sprague-Dawley rats (200-350 g; either sex), all killed by either cervical dislocation or overdosed (i.p.) with sodium pentobarbitone, were cleared of surrounding connective tissue, nerves and blood vessels under a dissecting microscope and placed in cold, carbogenated (95% v/v O_2, 5% v/v CO_2) Krebs solution (Kemp and Cocks, 1997). Human airway preparations (0.5-1 mm in external diameter) were dissected from lungs of cancer patients undergoing thoracotomy at The Royal Melbourne Hospital, Melbourne, Australia. The epithelium was removed from some bronchi either by mechanical abrasion of the luminal surface (guinea-pig) or by brief, gentle intraliminal flushing of the airways with Krebs containing 0.1% v/v Triton-X100 (mouse and rat). In each case, removal of the epithelium was verified histologically in 8 μm formalin-fixed, paraffin sections stained with haemotoxylin and eosin. Ring segments (approximately 2mm long) of bronchi and bronchioles were mounted in Krebs (37°C) on stainless steel wires (40 μm) in dual channel (5 ml) Mulvany-Halpern myographs (JP Trading, Aarhus, Denmark) to record changes in isometric force (Kemp and Cocks, 1997). After equilibration at a passive force between 0.2 g and 0.3 g, tissues were contracted to their maximum levels of active force (F_{max}) with acetylcholine (30 μM), thoroughly washed with Krebs and allowed to return to baseline. Various drugs or their vehicles were then added and 30 min later all tissues were contracted to approximately 40% F_{max} with titrated concentrations of carbachol (10-500 nM). The L-type voltage-operated Ca^{2+} channel inhibitor, nifedipine (0.3 μM) was added to all mouse and rat tissues after obtaining F_{max} to control characteristic phasic contractile activity with carbachol. When a stable level of active force to carbachol was obtained, tissues were exposed to cumulatively increasing concentrations of the PAR-1 and PAR-2-activating enzymes, thrombin (bovine serum, Sigma, MO, USA) and trypsin (bovine pancreas, 3x crystallised, Worthington Biochem, NJ, USA) respectively, and their synthetic tethered ligand peptide sequences, SFLLRN-NH_2 [<400>1] and SLIGRL-NH_2 [<400>2] (each >95% purity; Auspep, Parkville, Australia).

To assess the effect of enzyme-mediated receptor desensitisation on responses to the synthetic peptides, mouse bronchi were allowed to recover to their initial level of active force to carbachol following cumulative concentration-responses curves to trypsin (0.001-0.3 U/ml) or thrombin (0.001-0.3 U/ml) but with enzymes still present in the myograph chamber. When the force again reached a steady level, they were tested for desensitisation with maximum concentrations of trypsin and thrombin (0.3 U/ml). If no response occurred the tissues were then exposed to cumulative concentrations of either SLIGRL-NH_2 [<400>2] or SFLLRN-NH_2 [<400>1] (0.1-30 μM).

The time course and mechanism of PAR-2 resensitisation were determined in mouse bronchi either left untreated (time control) after acetylcholine washout or treated with trypsin (0.3 U/ml at 2 min intervals) over a period of 20 to 30 min. Tissues were then contracted with carbachol to approximately 40% F_{max} and exposed to trypsin (0.3 U/ml) at 0, 15, 45, 80 or 120 min after washout. Time controls to trypsin in non-desensitised tissues were not different at any of the times examined. The protein trafficking inhibitor, brefeldin A (10 μM) and the protein translation blocker, cycloheximide (70 μM), were then used to explore the mechanism underlying PAR-2 resensitisation following desensitisation with trypsin. In these experiments, trypsin-desensitised tissues were either left untreated (control) or treated with brefeldin A or cycloheximide before re-exposure to trypsin (0.3 U/ml) at 45 min.

EXAMPLE 2

In vivo studies

Male Sprague-Dawley rats (8 weeks) were anaesthetised (xylazine 10 mg/kg, ketamine 100 mg/kg and 50 mg/kg each 30 min thereafter, i.p.) and cannulae were placed in the trachea, carotid artery and jugular vein. Spontaneous breathing was stopped by an intravenous injection of pancuronium bromide (0.4 mg/kg and 0.2 mg/kg each 30 min thereafter) and rat were ventilated (tidal volume 8 ml/kg at 90 breaths/min, SAR-830 ventilator, CWE Inc., Ardmore, USA). Breath-to-breath measurement of airway resistance (R_L) and dynamic compliance (C_{dyn}) were calculated from flow and transpulmonary pressure recordings (PMS800, Mumed, London, UK). Flow was measured over the tracheal cannula (Fleisch

pneumotachograph, Lausanne, Switzerland) and transpulmonary pressure was measured with a differential pressure transducer, one end being connected to the outlet of the tracheal cannula, the other to an air-filled cannula inserted in the oesophagus. A rectal probe was used to monitor body temperature. Serotonin (5-HT; 0.3 mg/kg i.v.) was administered as a bolus dose at 5 min intervals until reproducible changes in R_L and C_{dyn} were obtained. Prior to each 5-HT challenge, lungs were hyperinflated once (by delivering twice the tidal volume) to prevent and reverse atelecasis. SLIGRL-NH_2 [<400>2], the scrambled peptide LSIGRL-NH_2 [<400>4] (both 0.1 mg/ml) and their vehicle controls (saline) were then delivered for 30 sec as aerosols generated by an ultrasonic nebuliser (AeroSonic 5000, DeVilbiss, Somerset, USA) in series with a second ventilator and the response to 5-HT determined 5 min later.

EXAMPLE 3

Data analysis

All cumulative responses (relaxations and contractions) were normalised as percentages of the initial level of active force to carbachol. Results are presented as mean ± s.e. mean and pEC_{50} (sensitivity) values were calculated by fitting concentration-response curves to a four parameter logistic function (Kemp and Cocks, 1997) using Graphpad Prism (version 2.0). Statistical comparison of mean pEC_{50} and maximum response (R_{max}) values were compared by two-tailed unpaired Student's *t*-tests or one way analysis of variance (ANOVA) with Tukey-Kramer's *t*-tests for multiple comparisons. $P<0.05$ was accepted as significant. Unless specified, all averaged data are from n>5 experiments.

EXAMPLE 4

Immunohistochemistry

Mouse

Fresh frozen, paraformaldehyde-fixed sections (14 μm) of mouse bronchus were incubated with a rabbit antiserum directed against the carboxyl-terminal of mouse PAR-2 (CSVKTSY [<400>5]) at a dilution of 1:500 for 48 h, washed with phosphate-buffered saline (PBS) and

then incubated with a biotinylated donkey anti-rabbit antiserum (Amersham) for 2 h, washed again with PBS and then labelled with FITC-conjugated streptavidin (Amersham) all at room temperature. After a final wash in PBS, the sections were mounted in buffered glycerol and viewed under a Biorad MRC1000 confocal scanning laser system installed on an Olympus IMT2 microscope with a krypton/argon laster. Visualisation of FITC was achieved using a 488nm excitation filter and a 522/535nm emission filter. Images of 768x612 pixels were then processed using Adobe Photoshop software. No staining was observed when the antiserum was preabsorbed with the immunising peptide sequence (10 μM at 4°C for 24 h).

EXAMPLE 5

Human

Paraffin sections (3 μm) were dewaxed and exposed to the rabbit anti-PAR-2 antiserum as described above. After 24 h exposure, a monoclonal mouse antibody directed against human trypsin (ogen) (Chemicon, MAB1482) was also applied. After a further 24 h exposure to both probes, binding of the rabbit anti-PAR antiserum was localised as described above, while trypsin(ogen) was localised using a donkey anti-mouse antiserum conjugated to rhodamine. The expression of PAR-2 and trypsin(ogen) was examined under epifluorescence using a Ziess Axioskop microscope equipped with separate filters for FITC and rhodamine fluorescence. Photographs were taken on Kodak Ectachome T160 film and subsequently scanned on a Macintosh computer using a slide scanner (Nikon). The separate images of FITC (green) or rhodamine (red) fluorescence were overlayed with Adobe Photoshop software, using obvious reference points to correctly align the images.

EXAMPLE 6

PAR-2 in airway epithelium

Using an antibody directed against the carboxyl terminal of mouse PAR-2 and confocal fluorescence microscopy, the inventors found specific PAR-2 immunoreactivity localised to epithelial cells, often focally within the cytoplasm, as well as to smooth muscle cells and

fibroblasts in the submucosa of the mouse bronchus (Figure 2). In functional studies, the mouse PAR-2 tethered ligand sequence, SLIGRL-NH_2 ([<400>2]; Nystedt *et al,* 1994) and trypsin each caused concentration-dependent, rapid onset and near-maximum relaxation of mouse bronchial rings contracted with the stable muscarinic agonist carbachol. These relaxations were abolished by either removal of the epithelium or inhibition of cyclooxygenase (Figures 1 & 2). For SLIGRL-NH_2 [<400>2] the sensitivity (pEC_{50}, -log M) was 5.6 ± 0.1 and the maximum relaxation (R_{max}) was 94 ± 3%. Similar concentration-dependent relaxations were obtained from the PAR-1 tethered ligand sequence SFLLRN-NH_2 ([<400>1]; Déry *et al,* 1988; λpEC_{50}, 5.6 ± 0.1; R_{max}, 76 ± 11%) and thrombin. In contrast to PAR-2 activation, removal of the epithelium or inhibition of cyclooxygenase unmasked smooth muscle contractions to PAR-1 activation with SFLLRN-NH_2 [<400>1] (Figures 1 & 2). Unlike SLIGRL-NH_2 [<400>2] (Blackhart *et al,* 1996) which is a specific activator of PAR-2, SFLLRN-NH_2 [<400>1] can activate both PAR-1 and PAR-2. However, the inability of SLIGRL-NH_2 [<400>2] to contract epithelium-denuded or cyclooxygenase-blocked preparations of the mouse bronchi indicates that SFLLRN-NH_2 [<400>1] causes smooth muscle contraction *via* activation of PAR-1. It is clear that the relaxations observed in response to SLIGRL-NH_2 [<400>2] or low concentrations of trypsin were due to activation of epithelial PAR-2 or an unidentified receptor with similar sensitivity to SLIGRL-NH_2 (<400>2) and trypsin. This is confirmed by the one observation that the responses to SLIGRL-NH_2 (<400>1) were abolished by prior desensitisation to trypsin but were unaffected by thrombin desensitisation whilst those to SFLLRN-NH_2 (<400>1) were abolished following desensitisation to both thrombin and trypsin.

Relaxations to SLIGRL-NH_2 [<400>2] and SFLLRN-NH_2 [<400>1] in the mouse bronchi were not due to nitric oxide (NO) since they were unaffected by the NO synthase inhibitor, N^G-nitro-L-arginine (100 μM) and the NO scavenger, oxyhaemoglobin (20 μM; Figure 2). Therefore, a prostanoid rather than NO mediated the relaxations of both PARs. PGE_2 is a likely candidate, since it is the most prevalent prostanoid released from the airway epithelium and the inventors found it to sensitively and powerfully relax mouse bronchi (pEC_{50}, 8.2 ± 0.1; R_{max}, 100%, Figure 1).

Smaller, intrapulmonary airways are likely to contribute more than larger airways to resistance to flow in the lungs. Therefore, the inventors investigated the effects of PAR-activating peptides in first generation branches of the mouse main bronchi. The inventors observed similar indomethacin-sensitive relaxations to the PAR ligands in these preparations although the sensitivity and maximum relaxation to both SFLLRN-NH_2 [<400>1] (pEC_{50}, 5.5 ± 0.02; R_{max}, 58 ± 10%) and SLIGRL-NH_2 [<400>2] (pEC_{50}, 5.1 ± 0.05; R_{max}, 58 ± 4%) were significantly less ($P<0.05$) than those in the main bronchi (Figure 2).

Since enzymatic activation of PARs is irreversible, rapid resensitisation mechanisms are critical for the maintenance of tissue responsiveness to PAR-activating proteases. Turnover of cloned PAR-1 expressed in selected cell lines has been shown to be rapid and dependent on both *de novo* synthesis of new protein as well as trafficking of performed receptors from intracellular pools (Dery *et al,* 1998; Bohm *et al,* 1996). The data generated herein show that in the mouse bronchi, PAR-2-mediated relaxations returned after 45 min following desensitisation to trypsin (Figure 3). This recovery was abolished by the protein trafficking inhibitor, brefeldin A (10 μM) or the translation inhibitor, cycloheximide (70 μM; Figure 3). These findings, together with the demonstration here that PAR-2 immunoreactivity was often localised in discrete cytoplasmic regions of airway epithelial cells (Figure 1), support the concept of rapid PAR-2 turnover from intracellular stores in airway epithelium. Furthermore, the inventors were unable to demonstrate specific localisation of PAR-2 mRNA in mouse bronchi using *in situ* hybridisation whilst readily detecting PAR-2 mRNA in the same tissue *via* reverse transcription-polymerase chain reaction. The apparent discrepancy between these findings could be explained by the immunohistochemical demonstration of intracellular stores of PAR-2 (Figure 1) which are continually replenished by translation of stable message of low transcript number. Thus, the capacity of airway epithelial cells *in situ* to rapidly recover their sensitivity to PAR-2 agonists following receptor desensitisation supports a role for epithelial PAR-2 in bronchoprotection.

In addition to the mouse, the inventors also observed PAR-mediated bronchorelaxation in the airways of other species. Thus, SLIGRL-NH_2 [<400>2] caused epithelium-dependent and indomethacin-sensitive relaxations in rat isolated bronchi (pEC_{50}, 5.5 ± 0.1; R_{max}, 56 ±

5%) and bronchioles (pEC_{50}, 5.1 ± 0.1; R_{max}, 67 ± 5%) and similar potency (pEC_{50}, 5.4 ± 0.2), epithelium-dependent relaxation in the guinea-pig isolated bronchi but with a significantly ($P<0.05$) lower R_{max} (31±5%) than those in both rat and mouse bronchi. Also, from experiments (n=4), the inventors observed PAR-2-mediated relaxations in human intrapulmonary airways which, although weak by comparison with those in mice, were blocked by indomethacin.

Importantly, the inventors have demonstrated here that SLIGRL-NH_2 [<400>2] is a highly effective inhibitor of bronchoconstriction *in vivo*. Thus, a 30 sec exposure to an aerosol of a 0.1 mg/ml solutio of SLIGRL-NH_2 [<400>2] , but not the scrambled peptide sequence LSIGRL-NH_2 [<400>4] caused inhibition (50-70%) of 5-hydroxytryptamine (5-HT)-induced changes in airway resistance (R_L) and dynamic compliance (C_{dyn}) in anaesthetised rats (Figure 4). This effect of SLIGRL-NH_2 [<400>2] could be functionally antagonised by higher doses of 5-HT.

It is clear from the data presented herein that the PAR-mediated bronchorelaxation described herein is cyclooxygenase-dependent. PGE_2 is the likely prostanoid involved since it is the only cyclooxygenase product released by airway epithelial cells capable of inducing potent bronchorelaxation. Also, substance P, another substance which induces epithelium-dependent bronchorelaxation, has been shown to mediate this response in the rat bronchi *via* release of PGE_2 from the epithelium. PGE_2 exerts other bronchoprotective actions in humans at concentrations well below those required for bronchodilatation. These include inhibition of cholinergic neurotransmission, lung mast cell activation, eosinophil chemotaxis, IL-2 production by T lymphocytes and IL-4-induced IgE production by B lymphocytes. Furthermore, PGE_2 synthesised by human airway epithelium probably contributes to refractoriness to histamine challenge in humans and exercise-induced asthma. Also, inhalation of PGE_2 in allergic asthmatics not only prevents the early phase of the response to allergen challenge but the late phase as well. Therefore, although inhalation of PGE_2 causes acute cough in man, stimulation of endogenous PGE_2 release by PAR-2 may place crucial roles in airway defence.

The studies presented herein assign functionality for PAR-2 and PAR-2 in the airways. Also, they show that PAR-2 activation results in powerful bronchodilatation *in vivo* and epithelium-dependent bronchial relaxation *in vitro* with no evidence for direct contraction. Therefore, airway epithelial PAR-2 is bronchoprotective. However, because PAR-2 is also expressed in the subepithelium, particularly on smooth muscle cells, the inventors propose a dual compartment model for the role of PAR-2 in the airways. In this model the barrier function of the epithelium separates epithelial cells (compartment 1) from the underlying tissues in compartment 2. Also, epithelial and subepithelial PAR-2 are differentially regulated by specific tryptic enzymes released preferentially in each compartment - epithelial trypsin for compartment 1 and mast cell tryptase for compartment 2. The inventors propose that trypsin is the endogenous activator of epithelial PAR-2 is supported by the demonstration here that trypsin(ogen) is co-localised with PAR-2 in human airway epithelium (Figure 5). In addition, trypsin is regulated by α_1-antitrypsin in the lungs whereas there are no known inhibitors of mast cell tryptase. Therefore, the model predicts that epithelial PAR-2 normally override any proinflammatory effects of PAR-2 activation in compartment 2 and that disruption of the epithelial barrier compromises the normal balance between the two compartments.

This study indicates that epithelial PAR-2 causes powerful bronchorelaxation *in vitro* and that their activation *in vivo* suppresses bronchoconstriction. Therefore, activation of PAR-2 initiates important paracrine protection in the airways by functionally antagonising elevated airway tone. If PGE_2 is the mediator of this effect, then airway epithelial PAR-2 have the potential to initiate other paracrine protective responses as well as autocrine protective effects within the epithelium. As such, these receptors offer scope fro new therapies for diseases like asthma and bronchitis. This is supported by the report by Gaurrea *et al,* (1999) which demonstrated that inhalation of PGE_2 in mild asthmatics markedly inhibited allergen-induced airway responses (constriction) and airway inflammation. The present invention provides a mechanism of activating the PGE_2-mediated bronchoprotection system.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood

that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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DATED this 12th day of February, 1999

The University of Melbourne

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

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PAR-2

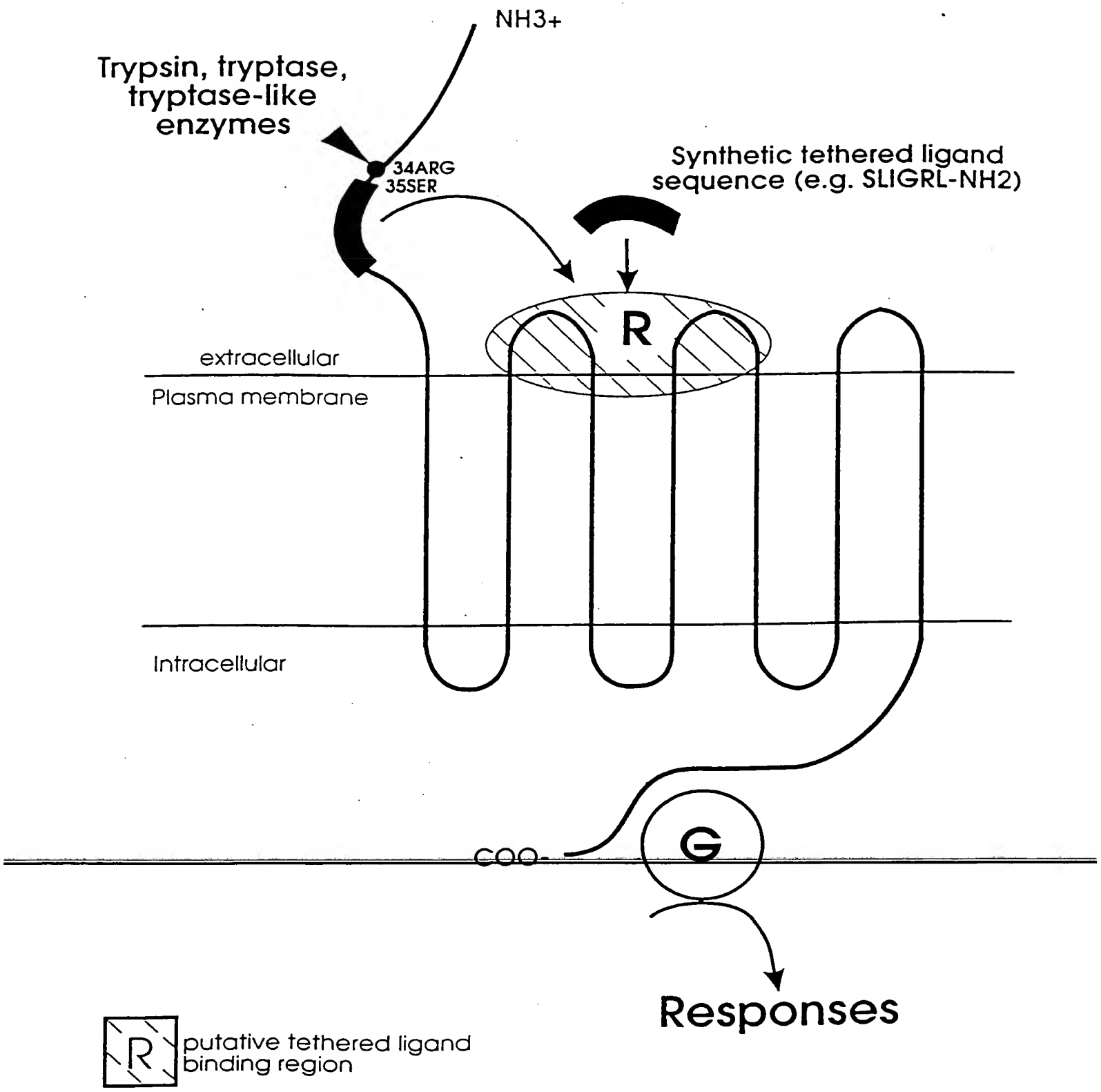
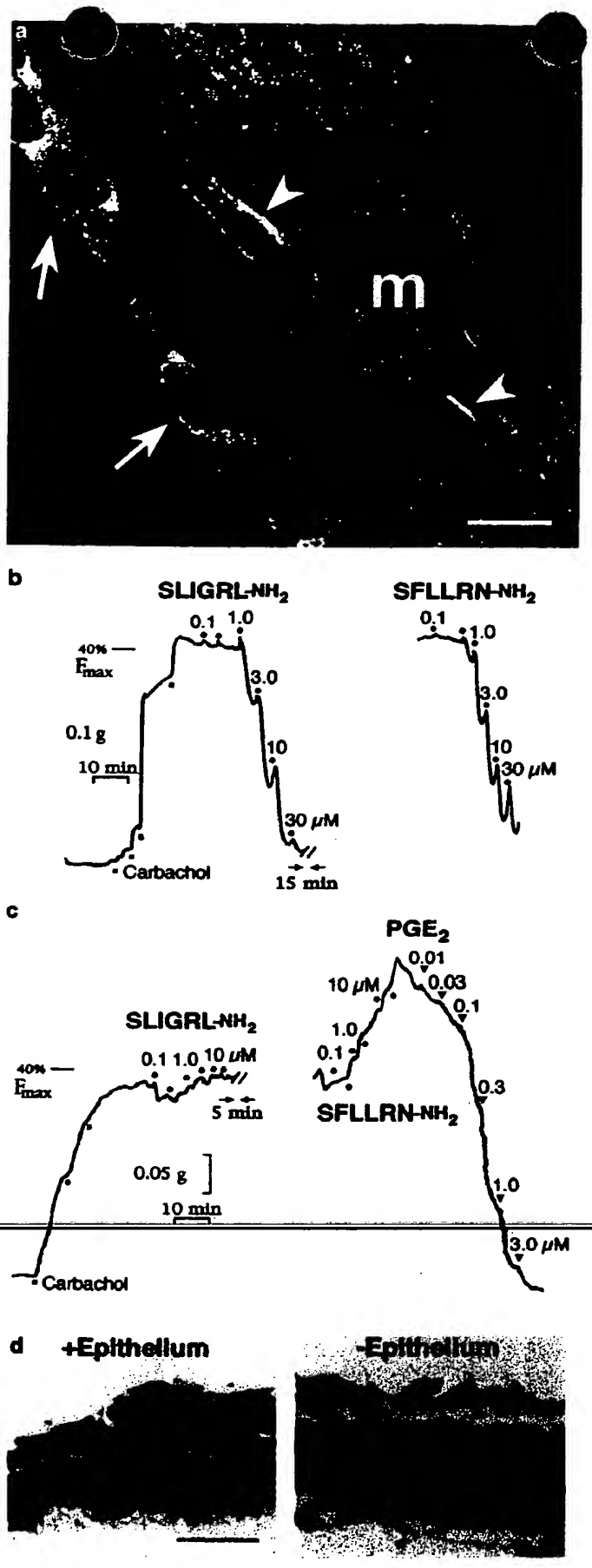


Figure 1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m
b
SLIGRL-NH2
SFLLRN-NH2
40% Fmax
0.1 g
10 min
0.1
1.0
3.0
10
30 μM
Carbachol
15 min
c
PGE2
0.01
0.03
0.1
1.0
10 μM
0.3
SLIGRL-NH2
SFLLRN-NH2
40% Fmax
0.1 1.0 10 μM
5 min
0.05 g
10 min
Carbachol
d
+Epithelium
-Epithelium

Figure 2

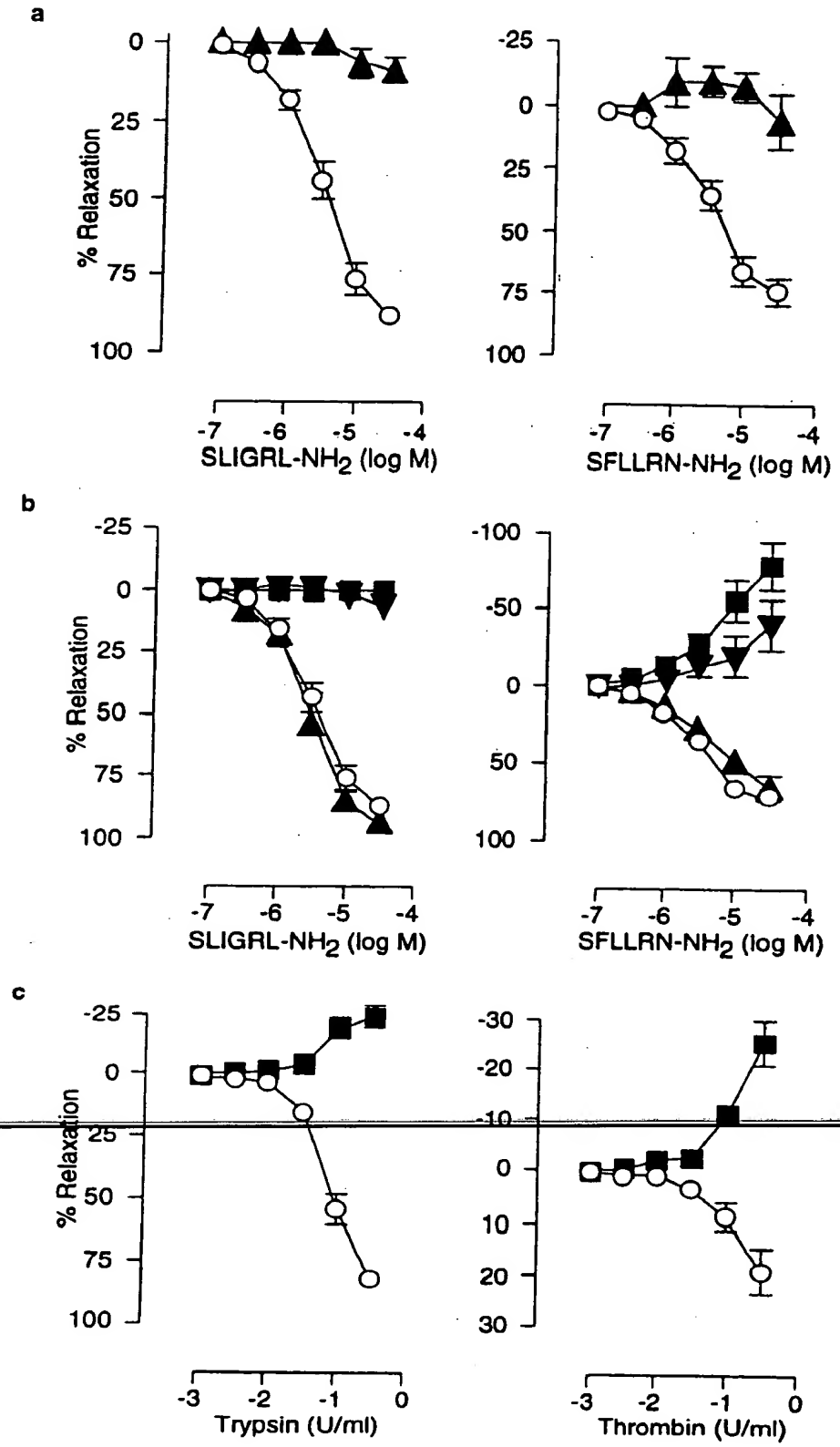
a
% Relaxation
0
25
50
75
100
-25
0
25
50
75
100
-7 -6 -5 -4
SLIGRL-NH2 (log M)
-7 -6 -5 -4
SFLLRN-NH2 (log M)
b
% Relaxation
-25
0
25
50
75
100
-100
-50
0
50
100
-7 -6 -5 -4
SLIGRL-NH2 (log M)
-7 -6 -5 -4
SFLLRN-NH2 (log M)
c
% Relaxation
-25
0
25
50
75
100
-30
-20
-10
0
10
20
30
-3 -2 -1 0
Trypsin (U/ml)
-3 -2 -1 0
Thrombin (U/ml)

Figure 3

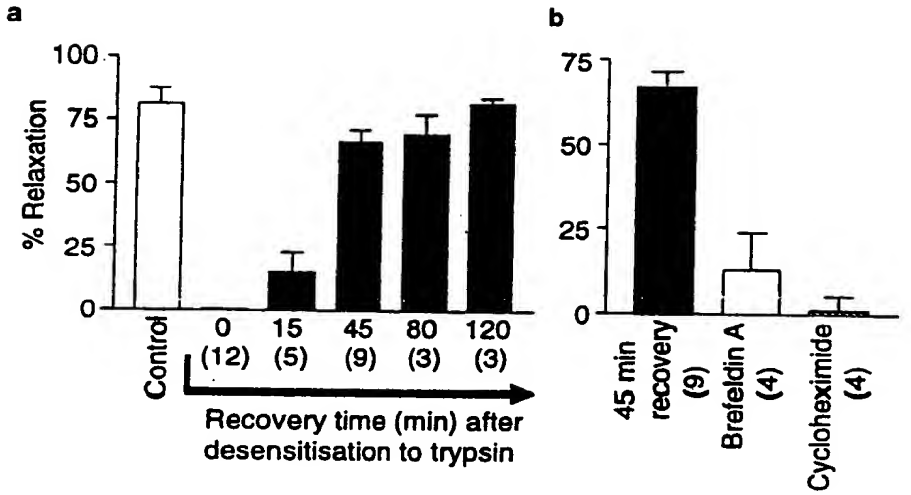
a
100
75
50
25
0
% Relaxation
Control
0
(12)
15
(5)
45
(9)
80
(3)
120
(3)
Recovery time (min) after
desensitisation to trypsin
b
75
50
25
0
45 min
recovery
(9)
Brefeldin A
(4)
Cycloheximide
(4)

Figure 4

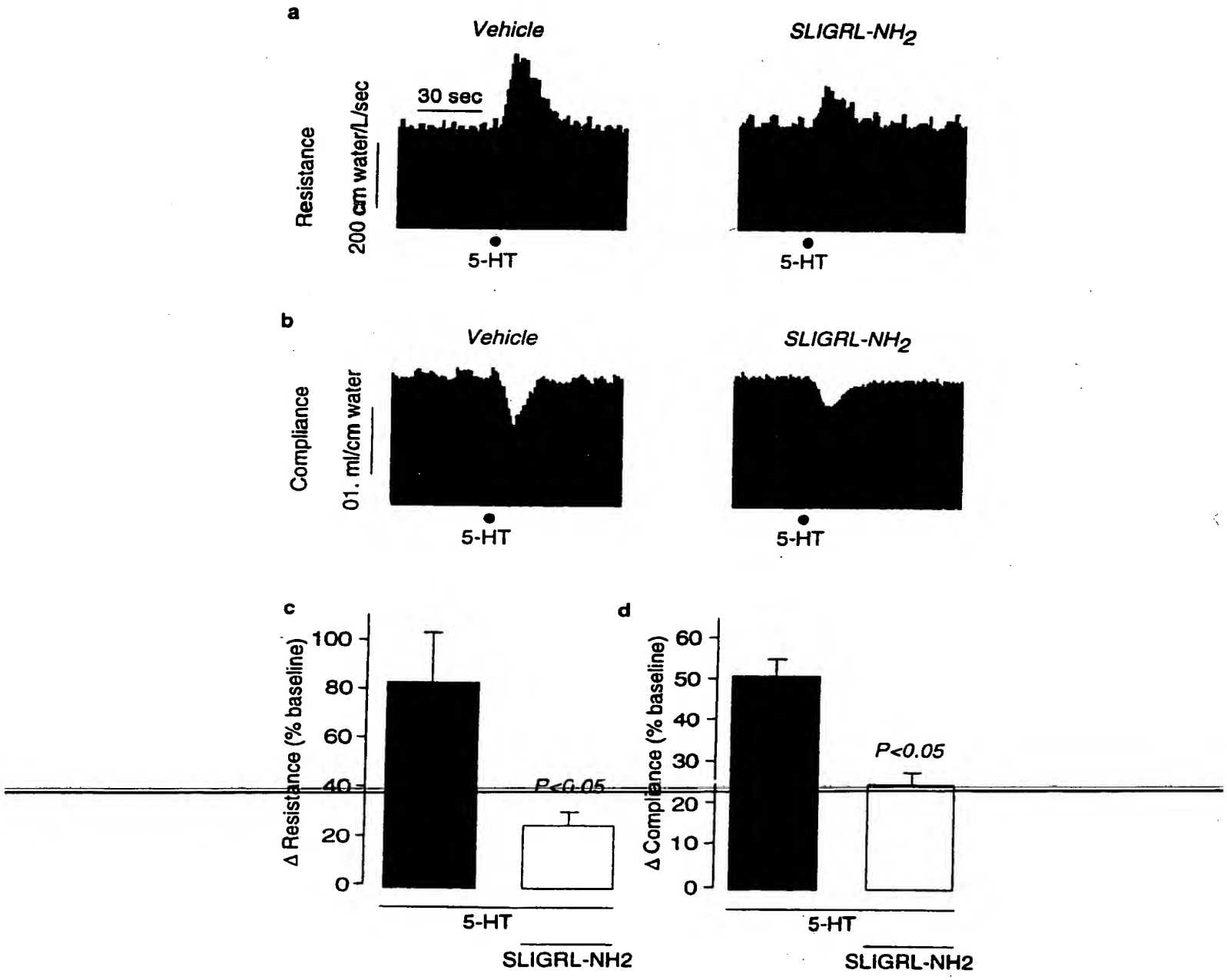
a
Vehicle
SLIGRL-NH2
30 sec
Resistance
200 cm water/L/sec
5-HT
5-HT
b
Vehicle
SLIGRL-NH2
Compliance
01. ml/cm water
5-HT
5-HT
c
Δ Resistance (% baseline)
100
80
60
40
20
0
P<0.05
5-HT
SLIGRL-NH2
d
Δ Compliance (% baseline)
60
50
40
30
20
10
0
P<0.05
5-HT
SLIGRL-NH2

Figure 5

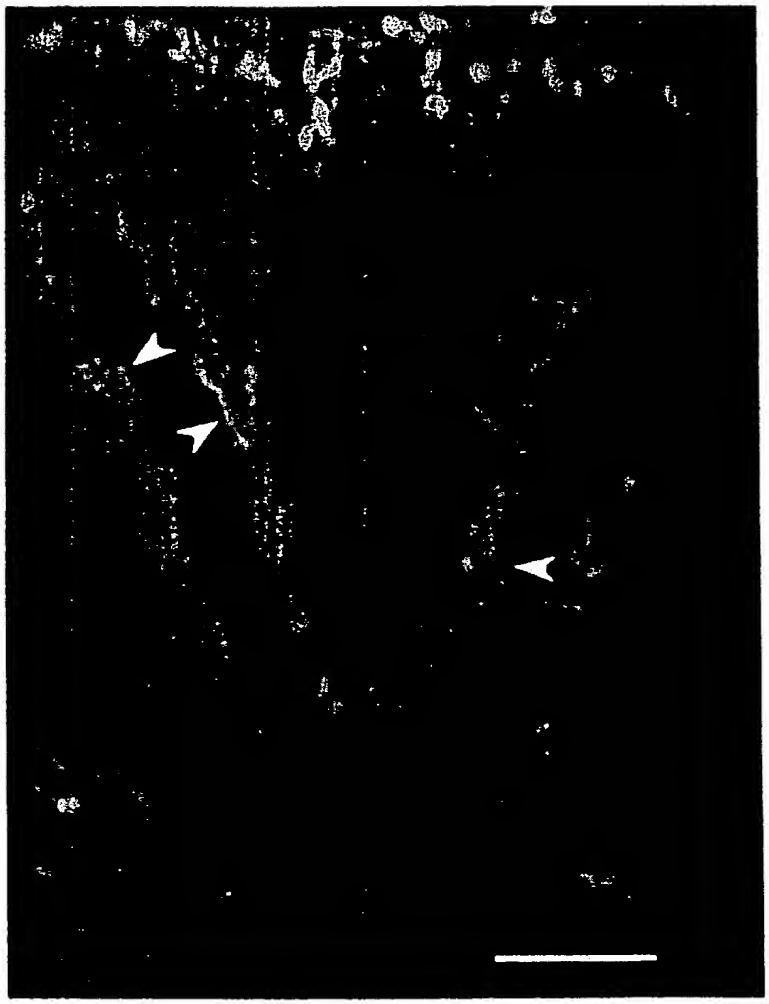

Figure 6